Poly(vinyl alcohol)-heparin biosynthetic microspheres produced by microfluidics and ultraviolet photopolymerisation

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Biosynthetic microspheres have the potential to address some of the limitations in cell microencapsulation; however, the generation of biosynthetic hydrogel microspheres has not been investigated or applied to cell encapsulation. Droplet microfluidics has the potential to produce more uniform microspheres under conditions compatible with cell encapsulation. Therefore, the aim of this study was to understand the effect of process parameters on biosynthetic microsphere formation, size, and morphology with a co-flow microfluidic method. Poly(vinyl alcohol) (PVA), a synthetic hydrogel and heparin, a glycosaminoglycan were chosen as the hydrogels for this study. A capillary-based microfluidic droplet generation device was used, and by varying the flow rates of both the polymer and oil phases, the viscosity of the continuous oil phase, and the interfacial surface tension, monodisperse spheres were produced from ~200 to 800 μm. The size and morphology were unaffected by the addition of heparin. The modulus of spheres was 397 and 335 kPa for PVA and PVA/heparin, respectively, and this was not different from the bulk gel modulus (312 and 365 for PVA and PVA/heparin, respectively). Mammalian cells encapsulated in the spheres had over 90% viability after 24 h in both PVA and PVA/heparin microspheres. After 28 days, viability was still over 90% for PVA-heparin spheres and was significantly higher than in PVA only spheres. The use of biosynthetic hydrogels with microfluidic and UV polymerisation methods offers an improved approach to long-term cell encapsulation. [http://dx.doi.org/10.1063/1.4816714]

I. INTRODUCTION

Hydrogel microspheres have been used in applications such as drug delivery,[1] enzyme encapsulation,[2] and cell delivery.[3] Hydrogels are particularly suited to these *in vivo* applications due to their high water content, good mass transport properties, and structural similarity to soft tissues in the body.[4] The majority of hydrogel microspheres used for cell encapsulation have been natural materials, such as alginate. However synthetic hydrogels, such as poly(vinyl alcohol) (PVA), have many advantages over these natural polymers including ease of purification, greater chemical and mechanical stability, reduced non-specific protein binding, reproducibility (minimal batch to batch variation), ease of modification and tunability.[5]

However, despite the favourable mechanical properties of synthetic gels, without modification they do not provide cellular recognition sites, which are important for cell encapsulation and delivery applications.[6] Therefore, the addition of biological molecules such as collagen or heparin to the synthetic network to form a biosynthetic hydrogel has become increasingly recognised as crucial in bulk cell encapsulation. Despite the recognition of the importance of biological signalling in cell differentiation and viability, the effect of adding biological molecules

on synthetic polymer performance has received minimal attention in the cell microencapsulation area. Heparin, a negatively charged glycosaminoglycan is a highly sulphated variant of heparan sulphate, which is known to be important in growth factor signalling to cells[7] and has been proposed to improve encapsulated cell viability long term.

Fabrication of hydrogel microspheres has traditionally used droplet extrusion techniques such as air or vibration assisted. More recently, Young *et al*. have reported on a novel electrospray method for producing synthetic hydrogel microspheres.[8] However the major issue with these techniques is the polydispersity of microspheres produced. Minimising the sphere polydispersity is particularly important for cell microencapsulation applications as size uniformity supports more controlled and predictable mass transport properties within the spheres, which is extremely important for cell function within the transplanted microspheres.[9]

Microfluidic methods have become increasingly common with the development and improvement of microfabrication processes and understanding of fluid flow at the microscale. Droplet microfluidics, has been used for many purposes, particularly in biomedical applications due to the potential for high throughput diagnostics and screening (lab-on-a-chip),[10,11] microreactors using picolitre volumes[12,13] as well as drug[14] and cell delivery applications.[15] Microfluidic droplet production is known to produce monodisperse spheres under gentle conditions.[16]

Microfluidic methods use an outer continuous phase to focus the flow of an inner prepolymer phase from which the spheres are fabricated.[17] Once droplets are produced they can be solidified using appropriate reactions. UV photopolymerisation is an effective and convenient way to crosslink droplets produced by microfluidic methods using functionalised polymers,[18–20] however, limited studies have been performed with hydrogels[21] and fewer again with synthetic or biosynthetic hydrogels.[22] The production of microspheres by coupling microfluidic droplet formation with UV photopolymerisation is a promising technique for use with biosynthetic hydrogel macromers and particularly for use in cell encapsulation. The microfluidic process itself is not harmful to cells and this has been exemplified in studies using alginate.[23–25]

Previous cell encapsulation studies using microfluidic droplet formation have been conducted using alginate as the hydrogel, which not only has the drawbacks of natural polymers as discussed above but it is also much more complex to induce gelation.[23–25] The gelation of alginate requires the presence of calcium ions, which are difficult to deliver through the oil continuous phase and therefore elaborate methods have been developed to deliver the ions once the droplets of alginate have been formed. These include attempts to merge droplets of calcium chloride with droplets of alginate[26] or encapsulating calcium carbonate particles and subsequently exposing droplets to acidified oil to release the calcium ions.[27–29] These processes have had mixed success and all involve additional steps. The use of a synthetic polymer such as PVA, which can be simply photopolymerised within the device would be able to avoid these complications. Co-flow microfluidics may provide a novel fabrication process for the microencapsulation of mammalian cells within biosynthetic spheres.

The use of microfluidics for cell microencapsulation in biosynthetic polymer based hydrogel microspheres has potential to produce monodisperse spheres, however, there has been little work done in the area. Therefore, this study will investigate the use of simple microfluidics for droplet generation with photopolymerisable poly(vinyl alcohol) (PVA) and heparin. It is hypothesised that a co-flow capillary microfluidic device can be used with a PVA macromer solution and a non-toxic continuous phase in combination with UV photopolymerisation under conditions compatible with cell encapsulation to produce monodisperse microspheres. The effect of process parameters including flow rates, continuous phase viscosity, and interfacial surface tension on the size of these spheres will be examined. Heparin, a biological glycosaminoglycan, will be introduced into the spheres and the effects on microsphere formation will be investigated. In addition, mammalian cells will be encapsulated in these spheres to assess the compatibility of the process with high encapsulated cell viability.

II. EXPERIMENTAL

A. Materials

All materials were from Sigma-Aldrich unless otherwise stated. PVA (13–23 kDa, 98% hydrolysed), heparin (sodium salt from porcine intestinal mucosa, 15 kDa), 2-isocyanatoethyl

methacrylate (ICEMA), glycidyl methacrylate (GMA) and sorbitan monooleate (Span 80) were used as supplied. Dimethyl sulfoxide (DMSO) and acetone were used as received from Ajax chemicals. 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959, Ciba Specialty Chemicals) was used as received. Phosphate buffered saline (PBS) was made up with 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic to pH 7.4. Food grade sunflower oil and castor oil were used.

B. Macromer preparation

PVA was modified with methacrylate groups according to a protocol by Bryant *et al.*[30] Briefly, PVA was dissolved at 10% (w/v) in DMSO at 60 °C under a nitrogen atmosphere until the PVA was completely dissolved. 2-isocyanatoethyl methacrylate was then added drop-wise under vigorous stirring, and the solution left to react for 4 h at 60 °C. The reaction was stopped by precipitating in toluene. The precipitate was re-dissolved in water and cleaned by ultrafiltration with a 10 kDa cut off membrane and then lyophilised to obtain a dry product. The degree of methacrylation was confirmed by H^1-NMR (300 MHz Bruker Avance DPX-300 Spectrometer) to be 7 crosslinkers/chain, by comparing the area under the peaks corresponding to the backbone (∼4.0 and 1.6 ppm) to the methacrylate groups (∼6.2 and 5.7 ppm).

Heparin was methacrylated according to a protocol by Nilasaroya *et al.*[7] Heparin was dissolved in PBS (pH 7.4) to make a 10% (w/v) solution. Glycidyl methacrylate (GMA) was added in equal molar amount as the number of moles of the disaccharide RU (1 mole heparin is made of ∼40 moles disaccharides). The GMA was added under vigorous stirring and the mixture was left to react, protected from light at room temperature for 14 days. The macromer was then precipitated in acetone to stop the reaction and dialysed against deionised water for 5 days in 10 kDa cut-off dialysis tubing and lyophilised on a Labconco freeze dryer to obtain a dry product. The degree of methacrylation was confirmed by H^1-NMR to be 3 crosslinkers/chain by comparing the peaks representing the methacrylate vinyl protons (5.7 and 6.1 ppm) to the peaks representing the protons on the disaccharide repeating unit (3.0–4.6 ppm).

Macromer solutions were prepared by dissolving 20 wt. % PVA-MA or 19 wt. % PVA-MA/1 wt. % Heparin-MA in PBS with 0.05 wt. % photoinitiator Irgacure 2959.

Oil solutions were prepared by thorough mixing of the desired amount of the surfactant Span 80 with the sunflower oil as shown in Table SI (supplementary material).[31]

C. Microfluidic device

The microfluidic device consisted of PTFE tubing, T-junctions and a fused silica capillary, with dimensions as shown in the schematic in Figure 1(a). The continuous phase was oil and its composition was varied in experiments as shown in Table SI (supplementary material).[31] The dispersed phase was the PVA or PVA/heparin macromer solution in PBS. A schematic diagram of the components of the system is shown in Figure 1(b). The PVA and oil were loaded into syringes and these syringes placed into syringe pumps (New Era). A long coil of transparent Tygon R-3603 tubing was connected to the outlet of the microfluidic device and this was positioned under a UV light (Bluewave 200, Dymax Co.) fitted with a rod lens attachment to give a uniform square of light (15×15cm) at 30mW/cm^2, 300–500 nm. This allowed enough time for polymerisation of the spheres while flowing through the tubing.

D. Microsphere formation and characterisation

The general procedure to produce microspheres involved simultaneously commencing flow of both the dispersed and continuous phases and allowing these to reach equilibrium (at least 5 min) so that the flow pattern and droplet size was stabilised. The UV light was turned on and spheres were collected in polypropylene centrifuge tubes to allow for separation from the oil phase. Microspheres were isolated from the oil by adding PBS to the centrifuge tube and centrifuging at 1000 rpm for 3 min. The oil was then aspirated and the pellet of spheres in PBS collected by pipette and resuspended in fresh PBS. This washing step was repeated twice.

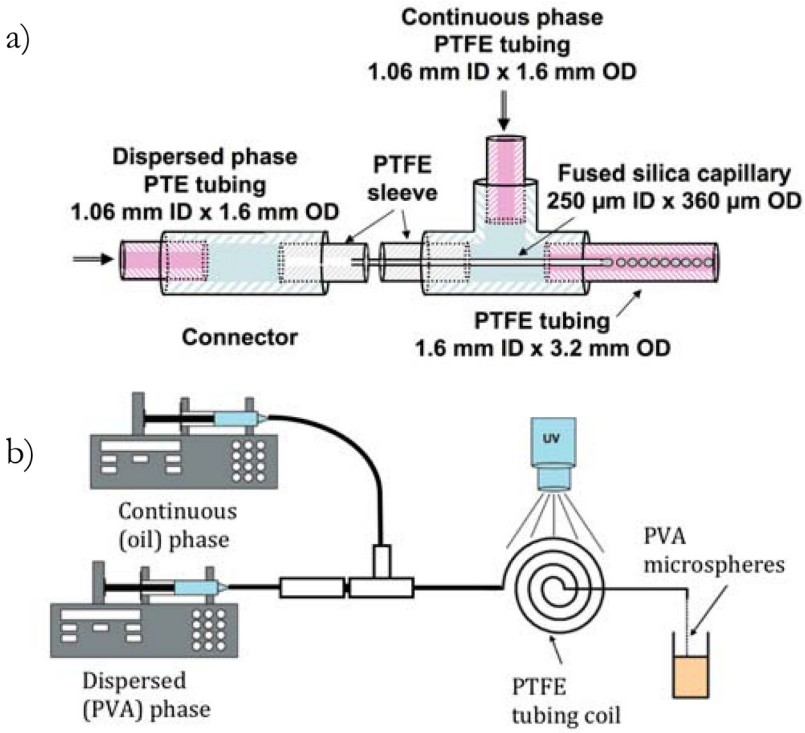

FIG. 1. (a) Schematic of microfluidic device used to produce the microspheres. (b) Schematic of the arrangement of syringe pumps, microfluidic device, tubing coil and UV light for the production of microspheres. Note: all tubing is level to ensure equal pressure due to height throughout system (continuous phase syringe pump shown above this level in the diagram for clarity only).

In the first part of this study, the device was characterised using PVA only. The oil flow rate was fixed at 1000 μl/min and the polymer flow rate varied from 0.1 to 50 μl/min. The interfacial tension was lowered by adding 0.5% Span 80.

Second, spheres from PVA/heparin were produced using the sunflower oil with 0.5% Span 80 as the oil phase.

Droplet generation modes were analysed by video microscopy. Spheres produced were incubated in PBS for 24 h after production to obtain equilibrium swelling. Microspheres were sized by microscopy, using the fluorescently labelled spheres to facilitate visualisation and allow better precision for particle sizing using ImageJ (v1.4, US National Institutes of Health). Sizing was also done using a Malvern Mastersizer 2000 for light scattering measurement of particle size distribution. Microspheres were imaged by brightfield optical microscopy for morphology.

E. Rheological characterisation of gelation

Photorheology was used to investigate the gelation of PVA and PVA/heparin hydrogels. An Ares 4400 rheometer (TA instruments) was used, with a modification to allow irradiation of the sample while measurements were being taken as shown in the schematic in Figure 2. A mirror at 45° to the upper plate was used to direct light down through a hollow shaft and through the upper quartz plate to the sample.

Both the elastic (G') and viscous (G'') shear moduli were calculated by the software from torsion measurements. For gelation time experiments, 20 μl of PVA or PVA/heparin macromer solution was dispensed onto the bottom plate (8 mm diameter). The upper plate was brought down to a final gap of 300 μm, and any excess macromer solution wiped away. A frequency of 10 Hz and strain of 10% was used for all samples. Dynamic time sweep test was started and then the UV light turned on and modulus evolution monitored.

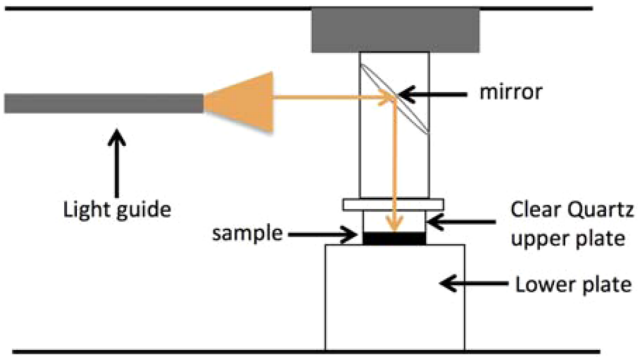

FIG. 2. Schematic of photorheometer setup. Light is directed from the light guide to the sample via reflection in the mirror through the clear quartz upper plate.

F. Mechanical characterisation

The mechanical properties of PVA and PVA/heparin spheres at equilibrium swelling were determined by uniaxial compression. Microspheres were produced at 2000 μl/min continuous phase and 10 μl/min dispersed phase flow rates. Individual spheres in a drop of PBS were placed on the lower plate of a rheometer equipped with a 10N load cell (Kinexus Pro, Malvern). Spheres were compressed between smooth, parallel flat plates at 10 μm/s until failure and the force-displacement data collected. Hertz theory was used in a similar manner to that demonstrated by Wang *et al.*[32] to determine the modulus (Eq. (1)). Briefly, the relationship between force (F), displacement (H), and modulus (E) can be determined from the following equation, where R is the initial radius of the sphere and ν is Poisson's ratio which was taken to be 0.5 as it has been calculated to be between 0.45 and 0.5 for PVA hydrogels[33]

$$F = \frac{4}{3}R^{\frac{1}{2}}\frac{E}{1-\nu^2}\left(\frac{H}{2}\right)^{\frac{3}{2}} = \frac{4}{3}R^{\frac{1}{2}}E^*\left(\frac{H}{2}\right)^{\frac{3}{2}}. \quad (1)$$

A least-square regression of F on $H^{3/2}$ was conducted and the slope consequently used to determine the modulus ($E^* = \frac{\frac{3}{4}2^{\frac{3}{2}}slope}{R^{\frac{1}{2}}}$) in a custom written program in MATLAB. Data were used from 0 to 10% deformation as Hertz theory is valid only for small strains.[32]

G. Cell encapsulation

L929 murine fibroblasts were cultured in Eagles Minimum Essential Media (EMEM) supplemented with 10% Fetal Bovine Serum and 2% Penicillin/Streptomycin. When required for experiments, cells were trypsinised and resuspended in DPBS. PVA-MA and PVA-MA/heparin-MA were dissolved in sterile DPBS and the cell suspension was added to give a final cell concentration of 1×10^6 cells/ml and 20 wt. % PVA or 19% PVA/1% heparin. Sterile photoinitiator I2959 was added to a final concentration of 0.05 wt. % and the solution gently mixed to ensure even cell distribution.

The cell/macromer solution was transferred to a syringe and microsphere formation proceeded as described previously. Encapsulation was performed with sunflower oil with 0.5% Span 80 and a continuous flow rate of 2000 μl/min and polymer flow rate of 10 μl/min. These conditions were previously identified to fabricate spheres ~400 μm diameter and with the highest flow rate, resulting in a higher production rate. Microspheres were separated from the oil as described previously, resuspended in complete EMEM and incubated at 37 °C in 5% CO_2. Viability of cells within the spheres was assessed at intervals up to 28 days by Live/Dead staining with calcein-AM and propidium iodide (PI), each at 1 μg/ml in DPBS. Encapsulated cells were incubated in the staining solution in the dark for 10 min, washed with DPBS and assessed

immediately by fluorescence microscopy (Zeiss Axioskop). Viability was expressed as the percentage of live cells compared to total cells.

H. Statistical analysis

All experiments were done with 3 replicates and the whole experiment was repeated 3 times. Thus a total of 9 samples were evaluated for each study. Values are reported as mean ± standard deviation, except for the sizing data, which is given as the mean ± standard error of the mean for 3 measurements on 3 batches of spheres. Two-way ANOVA was used to determine statistical significance for mechanical properties and cell viability using Minitab15 statistical analysis software.

III. RESULTS AND DISCUSSION

A. Microsphere formation with PVA only

Microsphere production by microfluidic methods is well known to be strongly dependent on the flow rates, viscosities, surface tensions, and droplet generation mode (dripping vs. jetting) and droplet size has been modelled by many groups to be a function of these parameters.[34,35] The device was characterised using PVA only initially by varying the above parameters.

1. Morphology

Microspheres exhibited a smooth, spherical morphology as shown in Figure 4(a). This morphology was expected as the emulsion of aqueous droplets in an oil phase relies on surface tension to pull droplets into a spherical shape to minimise surface energy.[36] In this method, droplets are allowed to form completely and relax into a spherical shape before polymerisation. The polymerisation mechanism does not disrupt droplet shape like some extrusion methods used with alginate,[37] and the droplets are completely polymerised within the coil of tubing before they come into contact with each other or come to rest in the collection vessel. Photopolymerisation with emulsion methods avoids many of the problems with extrusion and impact with a gelling bath.

A regular spherical morphology is particularly important for microspheres to be used in cell encapsulation. For applications where spheres are to be implanted *in vivo*, the surface geometry can impact on the host response. Teardrop shaped microspheres with "tails" or other irregularities, such as can be formed with some extrusion droplet generation methods, have been shown to suffer from significantly greater fibrotic encapsulation than smooth spheres.[38] This is particularly detrimental for cell encapsulation as the fibrotic capsule limits and can sometimes prevent diffusion of necessary nutrients leading to cell death.[39]

2. Size

As seen in Figure 3, with the PVA and oil combinations chosen this device produced spheres from 635–803 μm as the flow rate of the dispersed phase increased without surfactant in the oil phase. With the addition of surfactant, as the dispersed phase flow rate was varied the droplet size increased from 360 μm at 0.1 μl/min until it reached a plateau of 628 μm at 10 μl/min. Onset of jetting occurred at 40 μl/min with and without surfactant, which explains the slight reduction in size at that point in Figure 3.

The spheres produced in the dripping mode were monodisperse with a coefficient of variation (CV) of 2–6%. In the jetting mode, spheres were less uniform with a slightly wider distribution and a CV of 8–15%. Uniformity is very important for many microsphere applications and particularly cell encapsulation. Monodisperse microspheres result in more consistent encapsulation, better predicted release profiles of therapeutic products and ability to determine and inject the required number of cells for implantation.[9]

Therefore, in order to obtain the smallest, most uniform spheres for the remainder of the study, surfactant was used in the oil and flow rates were chosen to produce spheres in the dripping mode.

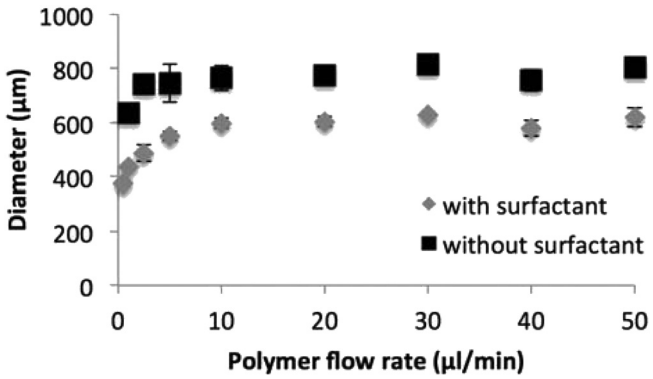

FIG. 3. PVA only sphere diameter as affected by surfactant with sunflower oil as the continuous phase with and without surfactant. Constant continuous flow rate of 1000 μl/min and a variable dispersed flow rate was used.

B. Addition of heparin

1. Morphology

Microspheres with heparin (Figure 4(b)) exhibited the regular, spherical morphology that was seen for pure PVA spheres, which is important for cell encapsulation and implantation. The "rings" that are seen on the PVA only spheres (Figure 4(a)) appear to be optical effects. The images in Figure 4 are phase contrast images and when viewed under plain transmission, the rings are not visible.

The most striking difference with the addition of heparin is the opaque nature and lack of homogeneity within the spheres. This observation suggests that phase separation may occur where the PVA is not completely miscible with the heparin. The immiscible nature of some GAGs with synthetic polymers has been noted by others.[40]

2. Size

The incorporation of heparin into PVA microspheres did not have a significant effect on size. As polymer flow rate increased so did the diameter of both PVA and PVA/heparin spheres, with no statistical differences observed ($p > 0.05$) (Figure 5). There were also minimal differences observed in the size distribution of PVA and PVA/heparin microspheres, as seen from the results of the laser scattering (Figure 6). The finding that heparin had negligible effect on size was expected as the fluid parameters that were shown to regulate droplet size (i.e., viscosity and interfacial surface tension) were unaffected by the small amount of heparin added to the macromer solution (see Table SI in the supplementary material[31]).

Viscosity was not statistically changed with the addition of heparin to the solution. Viscosity of polymer solutions is affected by molecular weight, concentration, and chain

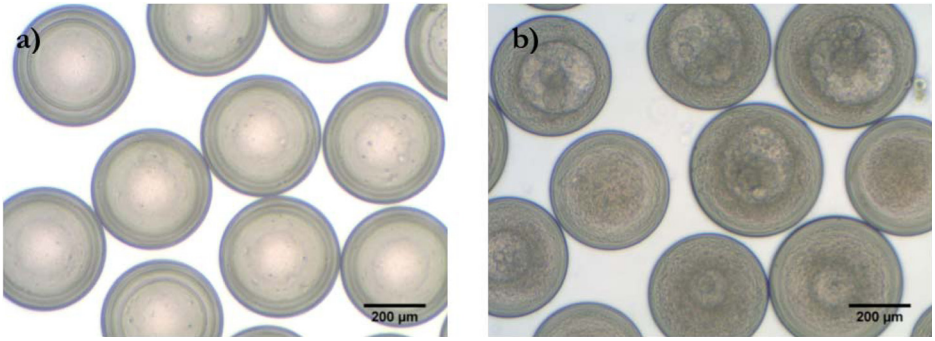

FIG. 4. Brightfield microscopy images of (a) PVA and (b) PVA/heparin spheres.

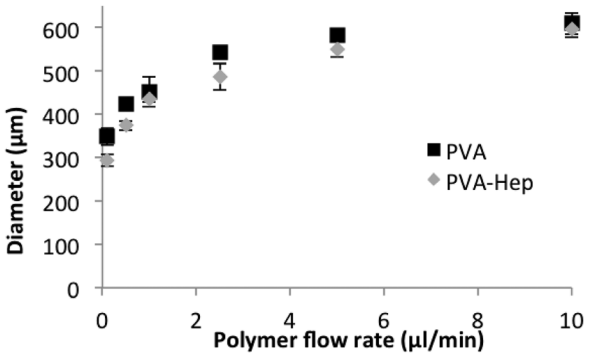

FIG. 5. Comparison of diameters between PVA and PVA/heparin microspheres showing that size was unaffected by the addition of heparin ($p > 0.05$). Error bars represent standard error of the mean for 3 batches of spheres, to show reproducibility between batches.

interactions.[4] The total macromer concentration was kept at 20% (i.e., 20% PVA vs. 19% PVA/1% heparin) and the average molecular weight of heparin is comparable to the PVA used (both ~16 kDa). The charged heparin molecules may affect the chain interactions in solution, however, the majority of the macromer is still PVA and the PVA-PVA interactions would likely dominate the behaviour. Therefore, it was expected that the viscosity would not be significantly altered.

Interfacial surface tension, another major factor in droplet formation, was not altered when heparin was included in the macromer solution (Table S1, supplementary material).[31] A biological polymer could interact with the PVA, oil or surfactant to affect the interfacial surface tension. However as PVA is uncharged, and heparin is charged but the surfactant is non-ionic, there are no major reasons why the interfacial surface tension would be expected to alter with the addition of heparin into the macromer solution. These results are important as it ensures the same parameters can be used for preparing PVA and PVA/heparin spheres of the same size and allows for direct comparison of properties such as mechanics and cell viability, which may be affected by droplet size.

3. Rheology

The PVA-MA/heparin-MA biosynthetic hydrogel system has been previously studied in bulk form by Nilasaroya *et al.*[7] In order to confirm similar gelation kinetics for PVA and PVA/heparin macromer solutions, rheological studies were conducted. Results shown in Figure 7 indicate that gelation begins immediately on exposure to light as seen in the rise in modulus. The cross over point of G' and G'', which is often used to estimate the gel point of the reaction,[41] occurred within the first few seconds of UV light exposure, which further emphasises

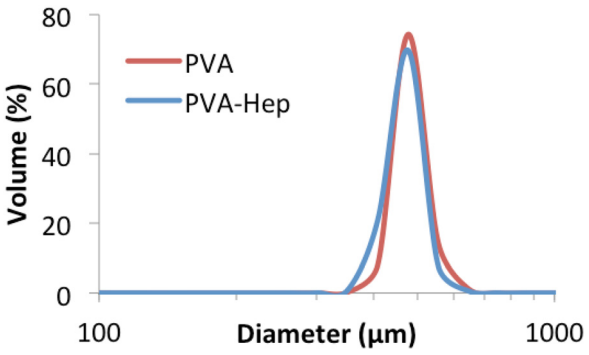

FIG. 6. Size distribution curves of PVA and PVA/heparin microspheres showing very similar distributions between the PVA and PVA/heparin spheres.

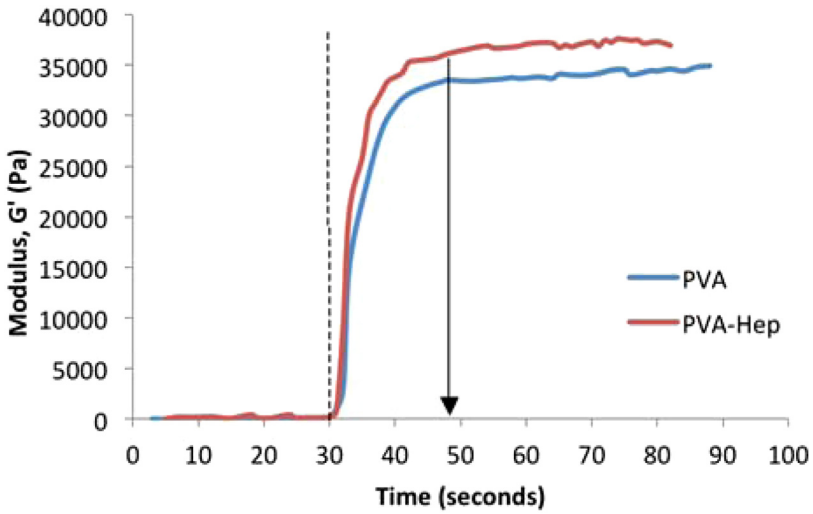

FIG. 7. Dynamic time sweep showing representative curve for PVA and PVA/heparin, showing very similar gelation profiles and final modulus. The dashed line indicates when the UV light is turned on. Arrow indicates complete gelation, i.e., plateau of modulus value. Note: G″ ranges from 0 to 100 Pa for both PVA and PVA-Hep for the entire period.

rapid gelation. $G' \gg G''$ which shows the elastic component dominates over the viscous component and indicates that a true gel forms. The evolution of G' has previously been correlated with reaction progression in photopolymerised hydrogels[42] and rheometry alone is often used to monitor gelation kinetics.[41] Martens *et al.*[43] demonstrated using near infrared spectroscopy (NIR) that PVA-acrylate photopolymerised hydrogels react to near 100% conversion, and the gelation kinetics observed with NIR correlate well with the results from rheometry for the current study.

Gelation appears to be complete within 15–20 s for both PVA and PVA/heparin, as evidenced by the plateau of modulus value. When polymerising the spheres however, it was observed that longer exposure to UV light was required due to the need for it to travel through the tubing and oil, which may cause diffraction, resulting in lower intensity at the droplet. The slight variation shown in final modulus values is within the range of test-to-test variability seen for either polymer.

These results are similar to those shown in previous studies which did not find any differences in gelation between bulk gels of PVA and PVA/heparin.[40] The methacrylate groups on the heparin have previously been shown to have similar reactivity to those on the PVA, although the heparin is methacrylated by glycidyl methacrylate rather than ICEMA.[40] The similar gelation kinetics are important for the incorporation of heparin into PVA spheres as it indicates that the biosynthetic spheres could be polymerised with the same exposure time as for pure PVA, and therefore the same conditions can be used for producing spheres from both macromer types.

4. Mechanical properties

The results from the mechanical testing of the microspheres (Table I) show that there are no significant differences between modulus values for PVA only and PVA/heparin spheres ($p > 0.05$). In addition, there are no significant differences between these values and the respective bulk gel modulus ($p > 0.05$) made from the same PVA or PVA/heparin macromer solutions.

These results are important on several levels. First, as the microspheres exhibit similar modulus values to the bulk gels, this indicates that the gel network formed in the microspheres is similar to the bulk gel. The modulus of synthetic, covalently crosslinked hydrogels is dependent on factors such as the molecular weight, crosslinks per chain, and macromer concentration.[4]

TABLE I. Compressive modulus of PVA and PVA/heparin spheres, compared to the bulk gel modulus.

	Modulus (kPa)	
Method	PVA	PVA/heparin
Microspheres	397 ± 47	335 ± 82
Bulk	312 ± 65	365 ± 72

Therefore, the properties of the microspheres could be easily tuned by varying these parameters of the macromer, as has been demonstrated for bulk gels.[4]

Importantly, the addition of heparin did not have a significant effect on the compressive modulus of spheres. This is supported by the findings of Nilasaroya *et al.*[7] who demonstrated that the addition of up to 2.5% heparin-MA did not affect the tensile modulus of bulk PVA/heparin hydrogels, compared to pure PVA gels. Pure heparin-methacrylate gels (20 wt. %, 3 crosslinkers/chain) were shown to be highly swollen and much weaker than PVA gels made from 20 wt. % macromer solution with 3 crosslinkers/chain. It was hypothesised that the highly charged heparin encouraged higher swelling due to repulsion between chains. Therefore the addition of heparin to the PVA in this study had the potential to increase swelling and therefore decrease the modulus of the gel spheres. However, the results indicate that the small amount of heparin added was not enough to significantly affect gel mechanics.

Appropriate mechanical properties are an important factor for hydrogel spheres for many applications. For cell encapsulation, the spheres must have a sufficiently high modulus and strength as it will dictate the resistance of the spheres to deformation and failure during implantation and when subject to forces at the implantation site. Failure of even a small percentage of the spheres could have catastrophic consequences for the whole graft when microspheres are implanted *in vivo*, as the release of transplanted cells into the host environment induces a significant immune response which can cause fibrotic encapsulation and subsequent death for the cells.[44] The modulus value range of 300–400 kPa for the spheres in this study compares well and is slightly higher than those reported for alginate spheres which vary in the range of 2–330 kPa (Refs. 32, 45, and 46) depending on the composition. Much effort has gone into increasing the mechanical strength of alginate based spheres by increasing the number of layers of alginate-PLL coatings[47] and also subsequent covalent crosslinking with chemical agents such as glutaraldehyde.[48] While all of these approaches have increased the strength to a degree, they all require multiple steps, which complicate the encapsulation process or introduce toxic crosslinking agents (e.g., glutaraldehyde), which have a detrimental effect on encapsulated cells. Therefore, the direct formation of synthetic, covalently crosslinked microspheres with inherent strength as demonstrated in the present study is an advance on previously reported approaches.

C. Cell encapsulation

The L929 cells were encapsulated in both PVA and PVA-heparin spheres with no obvious differences observed in the formation of spheres with the addition of cells. The encapsulated cells are seen to be well distributed throughout the PVA spheres (Figure 8(a)) and concentrated within the centre of the PVA-heparin spheres (Figure 8(b)).

These results show that the addition of cells did not disrupt formation process of spheres and that they retained the smooth spherical surface observed for the spheres without cells, which is critical for *in vivo* applications.

Cell viability, as assessed by Live/dead staining at 24 h post-encapsulation, was 89.9 ± 6.8% for PVA and 90.1 ± 5.9% for PVA-heparin spheres. This indicates that the encapsulation process itself had minimal effect on the cells. As seen in Figure 9, viability stayed high in both PVA and PVA-heparin spheres over the whole study period. However by 28 days cells in PVA-heparin remained over 90% viable, and had a significantly higher viability than cells in PVA only spheres ($p < 0.05$). These results show that the addition of heparin had a beneficial effect on long-term encapsulation viability.

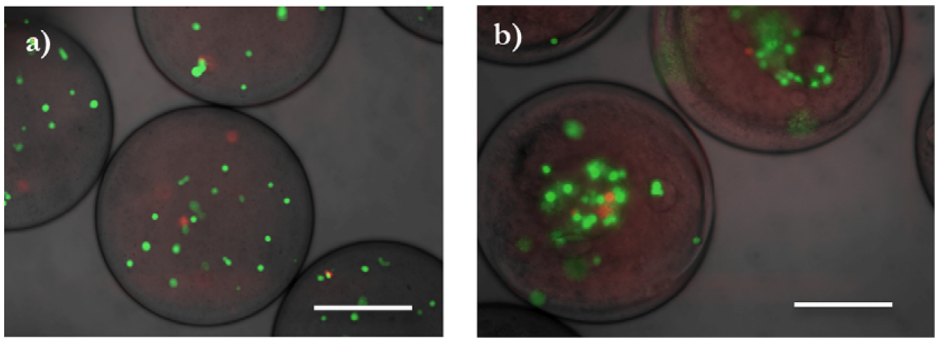

FIG. 8. Merged Live/Dead fluorescent image (live cells green, dead cells red) with brightfield image showing L929 cells encapsulated in (a) PVA and (b) PVA-heparin microspheres 24 h after encapsulation. Scale bar is 200 μm.

Cell viability is one of the critical characteristics for the application of cell encapsulation, as the viability of cells must be kept as high as possible for effective functioning of implanted spheres. The processes of UV photopolymerisation and microfluidics have been used separately for the encapsulation of cells and neither has been found to significantly affect encapsulated cell viability. The microfluidic process itself is reasonably benign with the cells not coming into contact with organic solvents and low flow rates resulting in low shear. Microfluidics has previously been used with alginate to encapsulate cells with high viability[23–25] and more recently with thiol-ene click reactions with PEG.[17] UV polymerisation is a common method for encapsulating cells in bulk gels and has been used with many cell types including chondrocytes[49] and mesenchymal stem cells[50] with high viability. Additionally, L929 fibroblasts that were encapsulated in the same PVA microspheres produced by a UV photopolymerisation and electrospray method also had >90% viability at 24 h.[8]

Heparin has been incorporated into bulk hydrogels for the purpose of sustained growth factor release and presentation to cells and was shown to have beneficial effects on viability.[51] Therefore, it is likely that the heparin in the gels in the current study acts as a reservoir for growth factors present in the media (from the FBS) or released by the cells in the gel and helps to present these to the cells and in this way helps to sustain the viability.

IV. CONCLUSION

This study has demonstrated an approach for the production of synthetic and biosynthetic hydrogel microspheres by combining microfluidic droplet generation with UV photopolymerization. Initial investigations using PVA showed the sphere size could be tailored by varying the

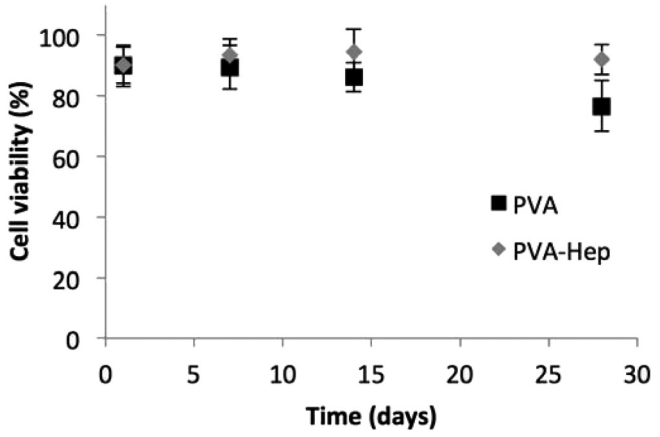

FIG. 9. Cell viability of L929 fibroblasts in PVA and PVA-Hep microspheres over 28 days. Heparin significantly increases viability over time ($p < 0.05$).

flow rates of the continuous and dispersed phases, the viscosity of the continuous phase, and the interfacial surface tension between the oil and PVA by the addition of surfactant. The microspheres produced by this method exhibited a smooth and spherical morphology and were uniform in size. The addition of the biological molecule heparin did not affect sphere morphology, size or mechanics. Importantly, this method was compatible with high encapsulated cell viability over 28 days in both PVA and PVA-heparin spheres, and cells retained a statistically significantly higher viability with the addition of heparin. These results show promise for future application to further cell types.

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